APPENDIX A - Protocol

STILLMEADOW
INCORPORATED

PROTOCOL For STUDY 24075-20

Test Substance:	BEHR Antibacterial Paint, #3190
Study Title:	SKIN SENSITIZATION: LOCAL LYMPH NODE ASSAY In MICE
Guideline:	OCSPP 870.2600
Test Facility:	STILLMEADOW, Inc. 12852 Park One Drive Sugar Land, TX 77478

Approved: Vincent A. Murphy — 04 Nov 20
Vincent A. Murphy, PhD, DABT — Date
Study Director, STILLMEADOW, Inc.

Approved: [signature] — 02 Nov 20
Management, STILLMEADOW, Inc. — Date

Reviewed: Kristina Rodrigue — 02 Nov 20
Kristina Rodrigue, RQAP-GLP — Date
Quality Assurance Director, STILLMEADOW, Inc.

Sponsor: BEHR Paint Company
1801 E. St. Andrew Place
Santa Ana, CA 92705
714 975 3127
jgilbert@behr.com

Approved: John a Gilbert — 11/03/2020
John Gilbert — Date
Chief R&D Officer

A. GENERAL

1.	Study Title:	Skin Sensitization: Local Lymph Node Assay in Mice
2.	Purpose:	To identify a skin sensitizing test substance or confirm test substance lacks significant potential to cause skin sensitization.
3.	Method Guidelines:	The study will be conducted according to US OCSPP 870.2600.
4.	Regulatory Compliance:	This study will be conducted in compliance with Good Laboratory Practice (GLP) standards: 1. EPA FIFRA 40 CFR 160 In the event of a regulatory inspection, Regulatory Inspectors will be provided with all study documentation requested. Sponsor will be notified of inspection of their study. All procedures in this protocol are in compliance with Animal Welfare Act Regulations. All methods can be found in STILLMEADOW, Inc. Standard Operating Procedures (SOP).
5.	Quality Assurance:	The Quality Assurance Unit (QAU) will review the protocol. Study information will be entered into the master schedule. In-progress inspection(s) will be performed to ensure integrity of the study. Any deviations from SOP, protocol or GLP standards will be reported to Study Director and Management. Raw data and report will be audited, and a statement prepared and signed which will specify dates inspections were made and findings reported to Management and Study Director.
6.	Test Substance:	BEHR Antibacterial Paint, #3190. Test substance identification should include name, lot/batch number and purity. Sponsor should also provide information regarding safety, storage conditions and disposal. Sponsor assumes responsibility for purity, stability, identity, synthesis methods and location of documentation.
7.	Positive Control Substance:	Alpha-Hexylcinnamaldehyde (CAS # 101-86-0), or alternate known sensitizer
8.	Proposed Schedule:	Testing should begin after test substance receipt, authorization to conduct study and study initiation. Proposed Experimental Start & End: 04 Nov 20 – 11 Nov 20 In-life portion: at least 6 days
9.	Study Director:	Vincent A. Murphy, PhD, DABT
10.	Experimental Summary:	Test groups (at least 3) and Vehicle Control group of mice will be treated once daily for 3 days, with appropriate solutions of test substance, or vehicle alone, to the dorsum of both ears. Positive Control group will be conducted concurrently, to confirm sensitization potential of animals used and validate procedures. All animals will remain untreated for 2 days after final dosing. The following day, all Test and Control mice will be injected with tritiated thymidine in the tail vein. Five hours later, animals will be sacrificed, and the draining auricular lymph nodes removed and prepared for cell suspension and examination. This regimen is based on Kimber, I, Hilton, J, Dearman, RJ, Gerberick, GF, Ryan, CA, Basketter, DA, Lea, L, House, RV, Ladics, GS, Loveless, SE, and Hastings, KL. Assessment of skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory exercise. Journal of Toxicology & Environmental Health, 53 563 - 79 (1998). Test substance will be considered a sensitizer (produce a positive response) if stimulation index (SI) is ≥ 3 together with consideration of dose response and, if/where applicable, statistical significance.
11.	Protocol Amendments:	Any protocol alteration will be justified, approved by Study Director, and recorded in writing.
12.	Sponsor Audits:	Sponsor may send authorized Representative to inspect test system and/or data on the STILLMEADOW, Inc. premises during normal working hours.

B. EXPERIMENTAL DESIGN

1. Animals

a. Species/Strain/Source:	Mouse / CBA-J / Jackson Laboratory; Bar Harbor, ME (or suitable supplier)
b. Species Justification:	The mouse is species of choice for a local lymph node assay to provide information on which human hazard can be judged.
c. Quantity & Sex:	5 per each Test group, 5 in Vehicle Control group, 5 in Positive Control group; females (nulliparous & non-pregnant) preferred
d. Age/Wt. at Initial Dose:	8 - 12 week / 17 - 35 g Weight variation shall not exceed ± 20% of mean
e. Animal/Group ID:	Tail marking / Cage cards
f. Acclimation & Health Status:	Animals will be acclimated for at least 5 days prior to initial dosing. Range-finding may be conducted during acclimation period. Normal weight gain, appearance and behavior will be factors used to select healthy naive animals for testing.

2. Animal Husbandry

a. No./Cage & Cage Type:	Up to 5 mice in polycarbonate box with bedding
b. Enrichment:	Provided to all animals during study
c. Food:	Teklad Global Diets® #2018, or equivalent, available ad libitum; analyzed by manufacturer for nutritional content
d. Water:	Tap water, available ad libitum, automatic system; municipal water supply analyzed by TCEQ Water Utilities Division
e. Contaminants:	There are no known contaminants in feed or water available to laboratory animals that would be expected to interfere with this study.
f. Environment:	Target temperature: 21° ± 3°C Target relative humidity: 30 - 70% 12-hr light/12-hr dark cycle (regulated automatically) Room ventilation: at least 10 air changes per hour

3. Dose Range-finding & Dose Selection: Prior to definitive study portion, a range-finder may be conducted, using at least 1 mouse/group, to determine the highest concentration that can be used while avoiding systemic toxicity and excessive local irritation. Concentrations generally will be in a series of 100% undiluted if liquid (or highest % suspension/solution attainable if solid), then 50, 25, 10, 5, 2.5, 1 and/or 0.5% dilutions. Doses selected will be 3 consecutive concentrations with top % as highest achieved without toxicity or excessive irritation.

4. Vehicle Selection: Vehicle should maximize test concentrations while producing suitable solution/suspension for test substance application. Preferred vehicles are acetone:olive oil (4:1 v/v), dimethylformamide, methyl ethyl ketone, propylene glycol and dimethyl sulphoxide. Others may be used; however, wholly aqueous vehicles should be avoided.

5. Test Substance Administration

a. Test Substance Application: Dosing order will be Vehicle control animals first, with no test substance or positive control substance in room; Test group animals second, with no positive control substance in room; Positive Controls last and in a cage rack separate from other groups. Vehicle and Test animals may be in different racks in the room, or if in same rack, Vehicle animals will be on shelf above Test animals. Test groups may share the same shelf. Care will be taken to avoid dosing near cage racks.

B. 5. a. (cont.)

Day 1: open application of 25 µL of appropriate test substance solution to dorsum of both ears, using a pipette and replacing tip between each group; if pipette is unusable due to consistency, substance will be applied via syringe
Day 2 & 3: repeat same procedure as on Day 1
Day 4 & 5: no treatment
Vehicle Control animals will be treated same as Test animals, but with vehicle alone instead of test substance. Positive Control animals will be treated in same manner, but with positive control substance alone or at least 25% in acetone: olive oil or other appropriate vehicle dilution.

b. Injection of Tritiated Thymidine:

Day 6: all Test and Control animals will be injected with 250 µL of phosphate buffered saline (PBS) containing 20 µCi of tritiated methyl thymidine via tail vein. At 5 hours after injection, animals will be sacrificed by CO_2 overdose, draining auricular lymph nodes excised, and pairs of lymph nodes from each individual animal processed.

c. Cell Suspension Preparation:

Single cell suspensions of lymph node cells from paired lymph nodes of individual animals will be prepared by gentle mechanical disintegration through 200 mesh stainless steel gauze. Cells will be washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C for at least 18 hours. Pellets will be resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid.

6. Observations

a. Body Weights:

Body weights will be recorded for each animal on Day 1 (prior to dosing) and Day 6, or time of discovery if found dead during study.

b. Clinical Observations:

All animals will be observed daily for clinical signs of toxicity; any dose site erythema will be graded (modified Draize technique) as follows.

None..... 0

Very slight (barely seen)...	1 = v	Moderate.....	3 = m
Well-defined.....	2 = s	Severe (beet red)	4 = e

Animals with significant signs of pain/distress will be given sufficient concentration of a suitable analgesic by subcutaneous or IM injection; analgesic will be readministered at appropriate frequency as needed. Any with signs of severe pain/distress considered irreversible will be humanely euthanized, per Study Director decision; remaining animals will be euthanized following final observation.

c. Determination of Tritiated Thymidine:

Incorporation of tritiated thymidine will be measured by scintillation counting as disintegrations per minute (DPM) for each group, expressed as DPM/lymph node.

7. Measurement & Calculation of Results:

Proliferative response of lymph node cells from paired lymph nodes of each animal is expressed as DPM per animal minus any background DPM. Group mean DPM with standard deviation is then calculated for each Test group and Control group. Final results are expressed as follows.

SI = mean Test DPM / mean Vehicle control DPM

In addition to SI calculations, statistical analysis (ANOVA) of data may be made. Analysis may include assessment of dose response relationship as well as pair-wise dosed group vs. solvent/vehicle concurrent controls, using appropriate method of statistical analysis (e.g., linear regression analysis and Dunnett's t-test). A single outlier value in a group may be identified using Dixon's Q test and removed from calculations per Study Director. If necessary to clarify results, consideration will be given to various aspects, such as if test substance has structural relationship to known skin sensitizers, if it is a significant skin irritant, and nature of dose response seen.

8. Data Interpretation:

Substance is regarded as a skin sensitizer (positive response) if at least one concentration of test substance results in $\geq$ 3x SI of Test group lymph node DPM vs. Vehicle Control lymph node DPM.

B. 8. (cont.)

However, to provide more complete evaluation of test substance, quantitative assessment may be performed by statistical analysis of individual animal data. Factors may include results of SI determinations, statistical analyses, strength of dose-response relationship, chemical toxicity, solubility, and consistency of Vehicle and Positive Control responses. Strong irritants may yield false positives due to initiation of significant lymphocyte proliferation; however, dose-response assay information may help uncover this situation. Concurrent evaluation of ear swelling also helps differentiate weak from strong irritants.

9. Test Substance Accountability:

A comprehensive inventory of test substance received and used will be kept. Test substance container(s) will be weighed when received at this facility, and all test substance use recorded. Test substance and test substance dosing solutions will be stored in original containers or equivalent, or in capped glass containers.

10. Unused Test Substance Disposal:

Unused test substance will be disposed of at Sponsor's expense after study termination.

11. Safety Precautions:

General safety precautions required by laboratory SOP will be followed. Sponsor will supply basic toxicity data on test substance to be used; however, since toxicity of test substances is often not well characterized, this laboratory will be conservative in setting safety procedures. Sponsor or Representative shall be notified of any exposure requiring physician's exam or care.

C. DATA MANAGEMENT

1. Records:

The following records will be maintained at STILLMEADOW, Inc. during the study, and archived upon study termination:

a. Protocol & protocol amendments (if any)
b. Final report & amendments (if any)
c. Study correspondence
d. Animal receipt/acclimation data
e. Test substance receipt, identification as supplied by Sponsor, preparation, administration, disposition; data on any vehicle used
f. Test animal information: number, species, strain, age, source, sex
g. Body weight data
h. Individual daily clinical signs & site erythema
i. Records from Control groups
j. Other pertinent data

2. **Data Storage:**

All raw data, originals of protocol, final report, any amendment(s) and a test substance sample will be archived at STILLMEADOW, Inc. for 15 years.

3. Data Reporting:

Final report will include following data as described in GLP standards:

a. Statement from QAU
b. GLP Compliance Statement & signature of Study Director
c. Names of scientific personnel involved in study
d. Dates of study initiation & termination
e. Identification, label information, description, storage of test substance, & identification of vehicle used
f. All pertinent animal data & husbandry, dosing information, observation methods
g. Reference to pretest range-finding if done
h. Description of test procedures
i. Individual daily observations for clinical signs of toxicity & any excessive site erythema
j. Determination of whether or not test substance was a sensitizer
k. Individual body weight data
l. Data & other identifying information from Control groups
m. Copy of this protocol; deviations (if any) & impact on study

4. Report Generation:

A final report will be generated after termination of in-life portion of the study; a draft report may first be issued for Sponsor approval.